Description

NOVEL ENZYME WITH DECOLORIZING ACTIVITY AND METHOD FOR DECOLORIZING DYES BY USING THE SAME

TECHNICAL FIELD

The present invention relates to a novel peroxidase enzyme with high dye degradation activity, the genetic information thereof and a method for degrading and decolorizing dyes by using the same.

BACKGROUND ART

Many of various synthetic dyes discharged from the processes of staining fiber products and from dyestuff production processes are slightly biodegradable substances, involving much difficulty in the degradation thereof in the nature. Because such colored wastewater is hazardous for the nature, regulations over the wastewater have increasingly been tightened.

In the fields of staining industry and dyestuff production industry, wastewater containing dyes has conventionally been treated, mainly by physical or chemical methods such as adsorption, concentration, chemical transformation and incineration. Although these treatment methods are efficient, these methods disadvantageously involve secondary pollution due to the generation of hazardous

byproducts and the discharge of the greenhouse effect gas via high-level energy consumption.

Recently, attention has been focused on a treatment method actively utilizing biotechnology with microorganisms or enzymes, as an alternative of the treatment methods. Several microbial strains capable of degrading dyes and colored substances have already been reported. For example, Phanerochaete chrysosporium as one species of white rot fungus is listed, which is known as one of lignin-degrading fungus.

However, all the dye-degrading microorganisms known so far have an activity to degrade only one or several types of dyes, so the ability of the microorganisms to treat dyes via degradation is naturally limited. Therefore, the development of an efficient method for treating of wastewater containing dyes has been desired.

Some of the present inventors have isolated a microorganism capable of degrading azo type- and anthraquinone type- dyes, namely Geotrichum candidum Dec 1 [which was internationally deposited at the National Institute of Bioscience and Human-Technology, the Ministry of International Trade and Industry, at 1-1-3, Higashi, Tsukuba, Ibaraki, Japan [transferred on February 17, 2000 from the original deposit (FERM P-15348); the accession number was FERM BP-7033] from the nature and have developed a method for degrading and decolorizing a wider range of dyes by microbial treatment

(Japanese Patent Laid-open No. 9-173051).

It has been assumed that the excellent ability of Geotrichum candidum Dec 1 strain to degrade dyes may possibly be based on the peroxidase activity of the fungal strain, but no instance of specific isolation or identification of such enzyme has been found. Hence, the genetic information thereof has absolutely never been elucidated.

The present invention has been attained toward the industrial demand as mentioned above. An object of the invention is to provide an enzyme applicable to more efficient treatment of wastewater containing dyes and a method for degrading and decolorizing dyes by using the enzyme.

The Geotrichum candidum Dec 1 strain exerts an activity to degrade a wide range of dyes and has also prominent enzyme stability. Therefore, the fungal strain per se or after immobilization on an appropriate carrier can be used for degrading dyes.

So as to enhance the industrial applicability, however, the treatment of wastewater containing dyes, particularly dye degradation should essentially be attained in an efficient manner economically.

It is useful for that purpose to use a dye-degrading enzyme owned by said microorganism through isolation and purification rather than to use the microorganism per se, to further elucidate the genetic constitution thereof to realize

the mass production of the enzyme and to use them in combination.

The present inventors have made investigations so as to attain the purpose. Because the novel fungus Geotrichum candidum Dec 1 strain exerts wide decolorizing spectra over various dyes, the inventors have made further investigations with their attention focused on the dye-degrading enzymes produced by the fungus. The inventors have successfully isolated and identified one of the enzymes, elucidated the gene encoding the enzyme and developed a mass expression system of the enzyme.

DISCLOSURE OF THE INVENTION

The first aspect of the invention is a peroxidase (sometimes abbreviated as DyP hereinafter) derived from Geotrichum candidum Dec 1 strain (FERM BP-7033), which has the following properties:

a) a property to degrade and decolorize dyes;

b) a molecular weight of 60 kDa, by the molecular weight assay using SDS-PAGE;

c) a molecular weight of 55 kDa, by the molecular weight assay using gel filtration; and

d) pI (isoelectric point) 3.8, by the assay by isoelectric focusing.

The second aspect of the invention is the enzyme in the first aspect, having the amino acid sequence of SQ ID NO. 7 in

the sequence listing.

The third aspect of the invention is the gene encoding the enzyme in the first aspect, having the DNA sequence of SQ ID NO. 8 in the sequence listing.

The fourth aspect of the invention is an expression plasmid vector comprising the coding gene in the third aspect.

The fifth aspect of the invention is a microorganism transfected with the expression plasmid vector in the fourth aspect.

The sixth aspect of the invention is a method for degrading and decolorizing dyes, which comprises using the enzyme in the first aspect or the microorganism in the fifth aspect for degrading and decolorizing dyes.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 depicts the results of the SDS electrophoresis of the enzyme DyP of the invention. In the figure, the left numerical figures show molecular weight, while the upper numerical figures 1 to 5 independently represent the molecular weight marker, crude enzyme solution, the enzyme solution after ion exchange chromatography, the enzyme solution after hydrophobic chromatography and the enzyme solution after ion exchange chromatography, in this order.

Fig. 2 depicts the results of the isoelectric focusing of the enzyme DyP of the invention. In the figure, the right

numerical figures show isoelectric points (pI), while the upper numerical figures 1 to 3 independently represent crude enzyme solution, purified DyP and the isoelectric point marker, in this order.

Fig. 3 is a graph depicting the relation between the enzyme activity of the inventive enzyme DyP and temperature.

Fig. 4 shows comparison between the primary structure in the proximity of the arginine (Arg) residue and the histidine (His) residue positioned at the center of the activity. In the figure, open square (□) shows the position of the proximal Arg residue, while open circle (○) shows the position of the proximal His residue and closed circle (●) shows the position of distal His residue.

BEST MODE FOR CARRYING OUT THE INVENTION

The invention is now described in detail hereinbelow.

The peroxidase of the invention is derived from Geotrichum candidum Dec 1 strain. The inventors isolated and purified the enzyme as follows.

[Preparation of culture broth]

According to general methods, Geotrichum candidum Dec 1 strain (FERM BP-7033) was cultured in a liquid culture medium. Any liquid culture medium of any composition can be used, as long as Geotrichum candidum Dec 1 strain can grow in the liquid culture medium. One preferable example is the potato dextrose

culture medium (sometimes abbreviated as PD hereinbelow) manufactured by Difco, Co., Ltd. So as to promote the induction of the intended enzyme, further, dyes may be added to the culture medium.

The cultivating conditions of the fungal strain may satisfactorily be determined in light of the type of the culture medium used. When the PD culture medium is selected, for example, the fungal strain is cultured at 15 to 37 °C, preferably at 30 °C for 3 to 8 days.

The culture broth thus recovered is subjected as a starting material for the purification of the dye-degrading enzyme to the following steps.

[Purification of dye-degrading enzyme]

The dye-degrading enzyme is now to be purified. The purification conditions are not specifically limited. For the purpose of the protection of the enzyme activity against inactivation, the culture broth is preferably handled at a low temperature, particularly in refrigerator.

Specifically, the microorganisms are first separated from the culture broth, from which the supernatant is recovered. In that case, separation processes such as filtration, centrifugation and membrane filtration may satisfactorily be used. Preferably, however, the microorganisms are removed via centrifugation, followed by filtration with glass filter. Contaminating polysaccharides are removed by performing the

combination thereof, to recover a crude enzyme solution at a high purity.

Subsequently, the recovered crude enzyme solution may be subjected to isolation, by using the dye-degrading activity described below as a marker. Prior to such isolation, however, concentration and desalting may be carried out so as to readily enable the separation, to prepare a concentrated crude enzyme solution.

Concentration may be done by methods for general use, for example ultra-filtration, salting-out, and evaporation. Preferably, concentration may be carried out by ultra-filtration. Additionally, desalting may be carried out by dialysis, ultra-filtration and electro-dialysis.

From the recovered concentrated crude enzyme solution can then be isolated the intended dye-degrading enzyme, by using the dye-degrading activity as the marker.

As the method therefor, ion exchange resin column chromatography, hydrophobic column chromatography, gel filtration column chromatography and the like may be used.

One of these column chromatography types or a combination of several types thereof may be used, to collect active fractions to isolate and purify the dye-degrading enzyme.

By the procedures, the inventors recovered the intended purified enzyme. The purified enzyme (205-fold active product) is at a single band by SDS-polyacrylamide gel

electrophoresis (sometimes abbreviated as SDS-PAGE hereinbelow). The enzyme is the dye-degrading enzyme, peroxidase in the first aspect of the invention. The inventors designated the enzyme DyP.

[Properties of the purified dye-degrading enzyme DyP]

The properties of the inventive enzyme DyP purified by the procedures were measured according to the following principles.

First, the molecular weight was measured by SDS-PAGE and gel filtration chromatography.

For the measurement by SDS-PAGE, a commercially available molecular weight standard kit for electrophoresis may be used as the molecular weight standard.

One example thereof includes Combithek manufactured by Boehringer Mannheim Yamanouchi, Co., Ltd. The kit comprises α-2-macroglobulin (molecular weight of 170 kDa), phosphorylase B (molecular weight of 97.4 kDa), glutamate dehydrogenase (molecular weight of 55.4 kDa), lactate dehydrogenase (molecular weight of 36.5 kDa), and trypsin inhibitor (molecular weight of 20.1 kDa).

As shown in Fig. 1 depicting the results of the measurement, the molecular weight of the inventive enzyme DyP is 60 kDa.

For the assay of the molecular weight of the enzyme by gel filtration chromatography, additionally, the inventors

used Sephacryl S-200 column and the standard molecular weight protein (manufactured by BIO-RAD, CO., LTD.).

Consequently, the molecular weight of the inventive enzyme DyP was assayed as 55 kDa.

Then, the isoelectric point of the inventive enzyme was measured. The isoelectric point was measured by isoelectric focusing.

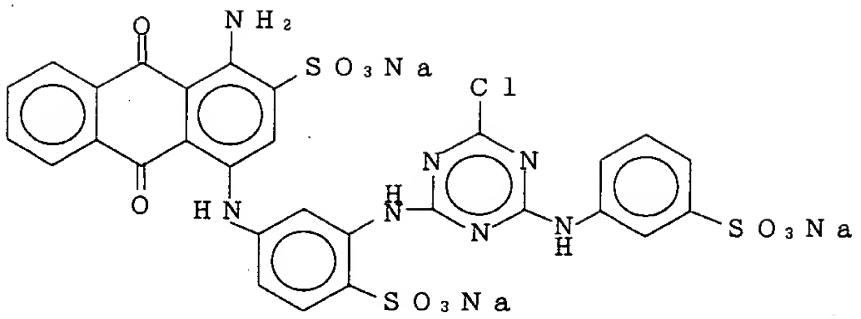
Consequently, the isoelectric point of the enzyme DyP was assayed as pI = 3.8, as shown in Fig. 2.

[Dye-degrading spectrum of dye-degrading enzyme DyP]

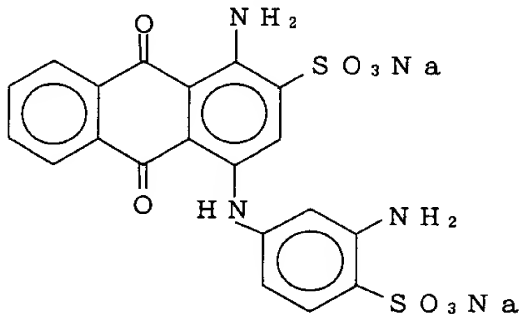
The dye-degrading enzyme DyP of the invention has an enzyme activity over azo type- and anthraquinone type- dyes, in particular, among dyes, and has an ability to degrade and decolorize these pigments.

The anthraquinone type dyes include for example Reactive blue 5, Reactive blue 19 and Reactive blue 114 (all manufactured by Nippon Kayaku Co., Ltd.); 1-amino-4-(3-amino-4-sodium-sulfonoanilino)-2-sodium anthraquinone sulfonate (sometimes abbreviated as AQ-1 hereinafter) and 1-amino-4-methylamino-2-sodium-anthraquinone sulfonate (sometimes abbreviated as AQ-2 hereinafter).

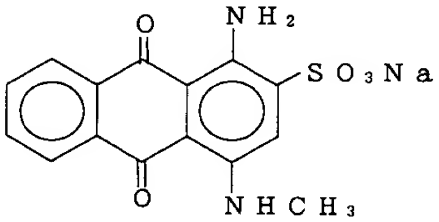
Herein, Reactive blue 5 is the compound represented by the following chemical formula.



AQ-1 is the compound represented by the following chemical formula.



AQ-2 is the compound represented by the following



Further, the azo type dyes include for example Reactive black 5, Reactive red 33, Reactive yellow 2 and Reactive blue 182 (all manufactured by Nippon Kayaku Co., Ltd.).

Other than the dyes, the dye-degrading enzyme DyP has an ability to degrade phenolic compounds such as 2,6-dimethoxyphenol and guaiacol, which are known as substrates for

manganese peroxidase (sometimes abbreviated as MnP hereinafter). As shown in the following examples, however, no effect of manganese compounds added to the reaction solution on the promotion of the enzyme activity is observed.

Alternatively, no reaction of DyP with veratryl alcohol known as a substrate of lignin peroxidase (sometimes abbreviated as LiP hereinafter) is observed.

As described above, surprisingly, DyP exerts substrate specificity different from those of MnP and LiP known so far. Thus, DyP can be said as a peroxidase differing from the known enzymes.

[Optimum reaction temperature of dye-degrading enzyme DyP]

The optimum reaction temperature of the inventive dye-degrading enzyme DyP is around 30 °C, as shown in Fig. 3. The enzyme DyP exerts stable dye-degrading activity within a temperature range of 15 °C to 35 °C. However, the enzyme activity rapidly decreases at a temperature above 35 °C.

[Temperature stability of dye-degrading enzyme DyP]

After the inventive dye-degrading enzyme DyP was stored at a fixed temperature for a fixed period of time, the ratio of the remaining activity was subsequently evaluated. Specifically, a solution of the dye-degrading enzyme DyP in 25 mM citrate buffer was stored at 30 °C or 40 °C for 14 days.

Consequently, the remaining enzyme activity of DyP was at 63 % when DyP was stored at 30 °C; and the activity was at

41 % when DyP was stored at 40 °C.

So as to compare the temperature stability with those of other peroxidases, solutions of individual enzymes in 25 mM citrate buffer were stored at 60 °C for 3 hours, by using a commercially available horse radish peroxidase (manufactured by Wako Chemical Co., Ltd.; sometimes abbreviated as HRP hereinafter) as the control. Subsequently, the remaining enzyme activities were compared to each other.

Consequently, it was shown that 65 % of the activity of the inventive dye-degrading enzyme DyP remained, but only 10 % of the activity of HRP remained.

The results show that the dye-degrading enzyme DyP has greater thermal stability, compared with currently known peroxidases.

The enzyme characteristics of the novel dye-degrading enzyme DyP in accordance with the invention are described hereinabove.

The dye-degrading enzyme DyP of the invention exerts a wider range of dye-degrading activity, compared with any of currently reported dye-degrading enzymes, and has also prominent enzyme stability, as apparently shown in the enzyme characteristics.

In the sixth aspect of the invention, hence, the use of the dye-degrading enzyme DyP enables efficient degradation and decolorization of such dyes.

So as to raise the industrial applicability of the dye-degrading enzyme DyP, essentially, efficient degradation of wastewater containing dyes and dyes should be attained economically.

One example includes a method using DyP after immobilization. The method comprises immobilizing DyP through adsorption or covalent bonding on immobilization carriers, such as ion exchange resin, synthetic polymer gel, naturally originated active charcoal and zeolite, and using the resulting DyP as bioreactor. For creating highly active bioreactor, the method is more useful than the method using the microorganism per se after immobilization.

As a means for more economically producing the enzyme, the gene encoding the intended enzyme is isolated, which is then introduced in a host microorganism capable of expressing the enzyme at a mass scale, so that DyP at a higher purity can be recovered more efficiently in a more stable manner.

Compared with the case of using the microorganism per se, a combination thereof enables the preparation of a bioreactor with a far more excellent cost performance.

[Schema of isolation of gene encoding dye-degrading enzyme DyP]

From the above respects, the inventors carried out the following procedures so as to obtain the genetic information of the DyP of the invention.

The concrete method for isolating the gene is

schematically described below.

First, partial hydrolysis of the inventive dye-degrading enzyme DyP purified by the aforementioned method was carried out by allowing trypsin (manufactured by Wako Chemical Co., Ltd.) to react with the enzyme.

The resulting five types of the partially hydrolyzed fragments were purified. Thereafter the amino acid sequences of the individual fragments were determined, to synthetically prepare the coding gene corresponding to each of the amino acid sequences.

Subsequently, PCR-amplified gene was recovered by PCR using the resulting coding gene as a primer and the cDNA derived from Geotrichum candidum as a template.

The resulting amplified gene was labeled by using the DIG labeling detection kit (manufactured by Boehringer Mannheim, Co., Ltd.).

By general methods using the labeled amplified gene as a probe, plaque hybridization with the Geotrichum candidum Dec 1-derived cDNA library prepared by using lambda phage λgt10, was performed.

From some hybridized colonies thus recovered were cut out the intended genes, which were then integrated into pUC18 plasmid, for subsequent sequencing. This was used as template for the following PCR.

[Determination of partial amino acid sequence of dye-degrading

enzyme DyP]

So as to prepare a primer for the gene encoding DyP, DyP was purified.

Purification of DyP can be performed by usual methods. The methods include for example a purification method comprising electroblotting from SDS-PAGE gel and a purification method by high-performance liquid chromatography (HPLC).

After the purified DyP was denatured by ordinary methods, partial hydrolysis thereof using trypsin was performed. Partially digested peptides thus formed were fractionated by HPLC. Consequently, five fragments were recovered. The amino acid sequence of each of the fragments was determined by the Edman method with a protein sequencer. Among the amino acid sequences of the resulting five fragments, the first sequence was Trp Lys. The amino acid sequences of the second and thereafter are shown in the sequence listing, where the second is shown in SQ ID NO. 1; the third is shown in SQ ID NO. 2; the fourth is shown in SQ ID NO. 3 and the fifth is shown in SQ ID NO. 4.

Among these amino acid sequences, a partial sequence (SQ ID NO. 5) of SQ ID NO. 3 and a partial sequence (SQ ID NO. 6) of SQ ID NO. 4 were selected as PCR primers.

[Probe preparation]

DNA encoding the two types of amino acid sequences was synthetically prepared by the following method.

By PCR using the resulting primer genes and the cDNA derived from Geotrichum candidum Dec 1 as PCR template, a first-stage gene amplification was practiced. Consequently, new 200-bp primers corresponding to the two primers were recovered.

Both the termini of the primers were subjected to T4 DNA polymerase treatment, to synthetically prepare plasmid-ligation sites. Then, the primers were ligated to the HincII site of pUC 18 as E. coli expression vector, to recover a recombinant plasmid.

The recombinant plasmid was amplified, by using E. coli JM 109 strain. From the resulting plasmid was cut out the coding gene. By a second PCR, the resulting DNA was sequenced (see the positions 1012 to 1181 of SQ ID NO. 8 in the sequence listing).

[Cloning of gene DyP encoding dye-degrading enzyme DyP]

From the Geotrichum candidum Dec 1 strain cultured separately was prepared RNA according to usual methods. From the resulting RNA was purified poly(A)$^+$RNA. Subsequently, the recovered poly(A)$^+$RNA was used to prepare cDNA with a cDNA kit (manufactured by TaKaRa).

After the recovered cDNA was subjected to ligation with T4 polynucleotide kinase kit, DNA of 1,200 to 2,000 bp was fractionated by electrophoresis.

Further, the DNA was inserted in the EcoRI site of lambda

phage λgt10, for packaging into the λ phage. The recovered phage was used for infection of E. coli.

Colonies hybridizing with the labeled probe previously prepared were screened. As a result, 11 candidates were obtained.

The results of the measurement described above indicate that DyP of the invention has a molecular weight of 60 kDa at a sugar chain content of 17 %, so the primary amino acid sequence is estimated to be 49.8 kDa. Additionally, the open reading frame of the gene encoding DyP is estimated to comprise 460 amino acids, namely 1380 bp.

Independently using the coding genes of the recovered 11 candidates, PCR was carried out again to evaluate the fragment size of the inserted cDNA. In other words, genes in the proximity of 1380 bp were screened.

Consequently, clone 92 carrying the cDNA of a 1600-bp size was recovered. The cDNA was cut out with BamHI from the recombinant plasmid, which was then integrated in pUC18. The resulting plasmid was designated pB92. It was verified that the clone 92 had a dye-degrading activity based on the dye-degrading enzyme DyP.

[DNA sequence of pB92 gene]

pB92 was sequenced with DNA sequencer. Consequently, it was found that the open reading frame of pB92 comprised 498 amino acids, namely 1494 bp and had a molecular weight of 53,306.

This indicates that pB92 carries the DyP gene. The amino acid sequence of DyP and the nucleotide sequence of the DyP gene, carried in pB92, are shown as SQ ID NOS. 7 and 8, respectively. In other words, DyP having the amino acid sequence described as SQ ID NO. 7 in the sequence listing is the enzyme described in the second aspect of the invention, while the gene having the nucleotide sequence described as SQ ID NO. 8 in the sequence listing is the gene in the third aspect of the invention.

Herein, the gene in the third aspect of invention (see SQ ID NO. 8 in the sequence listing) when modified with deletion, substitution, addition and the like in a part of the sequence is also encompassed within the scope of the invention, as long as the resulting modified gene has the same effects as those of the gene of the invention.

Further, pB92 as the plasmid vector carrying these genes is described in the fourth aspect of the invention.

Still further, a transformant recovered by transfecting E. coli with pB92 is described in the fifth aspect of the invention. When the transformant is used, the dye-degrading enzyme DyP of the invention can efficiently be produced.

The invention will now be described more specifically in examples hereinbelow. However, the invention is not limited to the examples.

Example 1

(Purification and properties of dye-degrading enzyme DyP)

[Purification of dye-degrading enzyme DyP]

150 mL of a PD culture medium (potato-dextrose culture medium, manufactured by Difco, Co., Ltd.) was placed in a 500-mL Erlenmeyer flask, into which 5 ml of the spore suspension of Geotrichum candidum Dec 1 (FERM BP-7033) strain was inoculated. Then, culturing was started. Culturing was continued at 30 °C and 120 rpm for 6 days.

After culturing, the culture broth was cooled to 4 °C and centrifuged at 7,200 × g for 20 minutes. 4,380 mL of the resulting supernatant was used for the following procedures.

The supernatant was filtered through a glass filter (GC50, manufactured by Toyo Roshi Co., Ltd.), to remove the polysaccharide contained therein.

Then, the filtrate was subjected to ultrafiltration on an ultrafiltration membrane (YM10) manufactured by Amicon, Co., Ltd., to concentrate the filtrate to 60 mL. The concentrate was dialyzed against 25 mM piperazine buffer (pH 5.5) and was then concentrated to 17.2 mL, by using Centriprep10 manufactured by Amicon, Co., Ltd.

The concentrate of 17.2 mL was charged on Super Q 650 M column of 2.8 × 6.0 cm (manufactured by Tosoh Co., Ltd.), which was preliminarily equilibrated with 25 mM piperazine buffer (pH 5.5). Subsequently, the column was rinsed with 200 mL of the same buffer, followed by elution on a linear gradient of 0 to 0.4 M.

Fractions with dye-degrading activity were collected and concentrated to 2.8 mL, by using Centriprep10 manufactured by Amicon, Co., Ltd. The concentrate was charged on Butyl Toyopearl of 1.6 × 6.5 cm (manufactured by Tosoh Co., Ltd.), which was preliminarily equilibrated with 25 mM citrate buffer (pH 5.5) and 0.8 M ammonium sulfate. Subsequently, the column was rinsed with 50 mL of the same buffer, followed by elution on a linear gradient of ammonium sulfate from 0.8 M to 0, to collect a fraction with the dye-degrading activity, which was defined DyP.

DyP was dialyzed against 25 mM citrate buffer, to recover purified DyP at 1.5 mg. The purified DyP solution was stored at 4 °C.

[Properties of dye-degrading enzyme]

The molecular weight and isoelectric point of the dye-degrading enzyme DyP recovered by the above procedures were measured.

The molecular weight was determined by SDS-PAGE electrophoresis and gel filtration method.

For SDS-PAGE electrophoresis, 10 % polyacrylamide gel and an electrophoresis apparatus of AE-6440 manufactured by Atto Co., Ltd. were used. As the molecular weight control, further, Combithek manufactured by Boehringer Mannheim Yamanouchi, Co., Ltd. was used.

Consequently, the molecular weight of DyP was assayed as

60 kDa.

For gel filtration, alternatively, Sephacryl S-200 column of 3.1 × 95 cm after equilibration with 25 mM citrate buffer (pH 5.0) was used, together with the standard protein kit manufactured by BIO-RAD Co., Ltd.

Consequently, the molecular weight of DyP was assayed as 55 kDa.

For measurement of isoelectric focusing, a low-pI calibration kit of Multiphor II 2-D for pH 2.5 to pH 6.5, manufactured by Pharmacia, Co. was used. Consequently, the isoelectric point of DyP was assayed as 3.8.

[Assay of dye-degrading activity]

The dye-degrading spectrum of the purified DyP was examined for nine types of dyes and three model compounds. The activity of the purified DyP to degrade these dyes or model compounds was assayed by measuring the degradation rates.

As the dyes, use was made of Reactive blue 5, 19 and 114; AQ-1 and AQ-2; Reactive black 5, Reactive red 33, Reactive yellow 2, and Reactive blue 182.

As the model compounds, additionally, use was made of 2,6-dimethoxyphenol, guaiacol and veratryl alcohol.

The dye-degrading activity was measured as follows.

0.2 to 0.4 mM aqueous hydrogen peroxide was added to a mixture solution of 3 mL of 25 mM citrate buffer (adjusted to the optimum pH for the degradation of each of the dyes)

containing each dye at a fixed pH (30 to 120 ppm) and 1 mL of 1.86 nM DyP solution, to initiate the enzyme reaction. Reaction was performed at 30 °C for a fixed period of time, to assay the reaction rate.

1 U of the dye-degrading activity was defined as the activity to decolorize 1 μmole Reactive blue 5 or AQ-2 for one minute. The results are shown in Table 1.

Table 1

(DyP activity to degrade each dye and model compound)

Color index	Chromogen	λmax	Optimum pH	Initial concentration (ppm)	Decolorizing activity (ppm/min)
Reactive blue 5	AQ	600	3.2	100	19.8
Reactive blue 19	AQ	590	3.2	70	13.1
Reactive blue 114	AQ	620	4.0	100	7.8
AQ-1	AQ	600	3.2	60	5.4
AQ-2	AQ	635	3.0	50	19.5
Reactive black 5	AZ	598	3.2	30	0.1
Reactive red 33	AZ	500	3.2	50	0.4
Reactive yellow 2	AZ	390	3.2	100	0.5
Reactive blue 182	AZ	610	4.0	120	20.9

As to 2,6-dimethoxyphenol used as a model compound, alternatively, absorbance at 470 nm was colorimetrically measured, which emerged via oxidation.

That is to say, a mixture solution of 2.79 nM DyP and 0.2 mM 2,6-dimethoxyphenol was reacted with 25 mM citrate buffer

(pH 4.5) containing 0.2 mM hydrogen peroxide.

As to guaiacol, 1 mM guaiacol was used in place of 0.2 mM 2,6-dimethoxyphenol, for absorbance measurement at 465 nm.

The results about the model compounds are shown in Table 2.

Table 2

(DyP activity to degrade model compounds)

Compound	Group	pH	Initial concentration (mM)	Oxidation rate (ΔOD/min)
2,6-Dimethoxyphenol	phenolic	4.5	0.2	0.29
Guaiacol	phenolic	4.0	1.0	0.29
Veratryl alcohol	nonphenolic	-	0.5	ND

The results in Table 1 indicate those described below.

The dye-degrading enzyme DyP exerts a high activity to degrade the anthraquinone type pigments. Specifically, the enzyme exerts an excellent degradation activity over Reactive blue 5, Reactive blue 19 and AQ-2, so the enzyme can efficiently degrade these pigments.

Additionally, the enzyme exerts an activity to degrade the azo type pigments. The enzyme efficiently degraded Reactive blue 182, in particular. The enzyme has an ability to degrade other azo type pigments, Reactive black 5, Reactive red 33 and Reactive yellow 2.

This apparently demonstrates that DyP has an action to degrade anthraquinone type pigments and azo type pigments.

Alternatively, the DyP activity over the model compounds is as follows, on the basis of the results in Table 2.

First, 2,6-dimethoxyphenol and guaiacol having phenolic hydroxyl group could efficiently be degraded by DyP. Alternatively, DyP could never degrade veratryl alcohol known as a substrate of lignin peroxidase.

This apparently indicates that DyP has a specifically high enzyme activity over the compounds having phenolic hydroxyl group.

[Optimum temperature of dye-degrading enzyme DyP]

The optimum temperature of DyP was determined by examining the decolorizing (degrading) activity of Reactive blue 5 at a fixed temperature. The results are shown in Fig. 3.

Fig. 3 indicates that DyP exerts a high peroxidase activity within a range of 20 to 35 °C, and also indicates that the optimum temperature is 30 °C.

Example 2

[Effect of metal ion on the activity of dye-degrading enzyme DyP]

Each 5 mM ions of calcium, zinc, copper (divalent), potassium, iron (divalent) and sodium were concurrently present in a reaction solution comprising DyP and 100 ppm Reactive blue 5, so as to examine the effects of these metal ions on the relative activity of the DyP enzyme.

The results are shown in Table 3.

Table 3

(Influence of metallic cation on DyP activity to degrade dyestuff)

Metal ion	Concentration (mM)	Specific activity (%)
No addition	-	100
Ca^{++}	5	81
Zn^{++}	5	69
Cu^{++}	5	75
K^{+}	5	81
Na^{+}	5	81
Fe^{++}	0.2	50

Table 3 shows that the enzyme exhibited a relative activity of about 80 %, when the ions were added, compared with the case of no addition. Particularly, the divalent iron ion concurrently present at 0.2 mM inhibited the activity at 50 %.

This indicates that the concurrent presence of the metal ions affects adversely the enzyme activity of DyP.

Example 3

(Determination of gene encoding DyP and the amino acid sequence thereof)

[Determination of partial amino acid sequence of dye-degrading enzyme DyP]

According to the Laemmli method (Laemmli, U.K. Nature (London), 227, 680-685 (1970)), Geotrichum candidum Dec 1 strain (FERM BP-7033) was subjected to SDS-PAGE, to separate purified DyP.

Subsequently, the DyP was electroblotted on polyvinyl difluoride (sometimes abbreviated as PVDF hereinafter) membrane according to the Towbin method (Towbin, H., Staehelin, T., and Gordon, J. Proc. Natl. Acad. Sci. USA, 76, 4350-4354 (1979)).

The PVDF membrane was treated with Coomassie Brilliant Blue (CBB-250), from which was then cut out solely the membrane corresponding to the stained band portion of the DyP. The band was then transferred in a 1.5-mL test tube. 50 μL methanol was added to the test tube, followed by addition of 200 μL reductive buffer (buffer, pH 8.5, containing 8 M guanidine hydrochloride salt, 0.5 M Tris buffer, 0.3 % ethylendiaminetetraacetate disodium (EDTA-2Na) and 5 % acetonitrile), for gradual shaking, from which the reductive buffer was removed.

Then, 50 μL reductive buffer containing 1 mg dithiothreitol was added onto the protein on the PVDF membrane, which was then left at 25 °C for one hour. After the PVDF membrane was transferred in a 200-mL conical beaker, followed by sequential rinsing individually with 100 mL water for 5 minutes, 100 mL 2 % acetonitrile for 5 minutes and 100 mL 0.1 % SDS for 5 minutes.

Thereafter, the PVDF membrane was transferred into a fresh 1.5-mL test tube, followed by addition of 500 μL polyvinylpyrrolidone PVP-40 (sometimes abbreviated as PVP-40 hereinafter) containing 1 mg methionine according to the

Iwamatsu method (Iwamatsu, A. Electrophoresis, 13, 142-147 (1992)), and the resulting mixture was left to stand at ambient temperature for 30 minutes.

After the PVDF membrane was additionally rinsed with 100 mL 10 % acetonitrile solution, it was rinsed three times with 500 μL of degradation buffer (100 mM ammonium bicarbonate, 10 mM calcium chloride, pH 7.8). Then the rinse solutions were discarded. Continuously, 500 μL of the same degradation buffer except for the content of 1 pmol trypsin was added, and then enzymatic reaction was carried out at 25 °C for 12 hours.

After the oligopeptide eluted from the PVDF membrane into the reaction solution was freeze-dried, the freeze-dried oligopeptide was then dissolved in 100 μL degradation buffer, followed by elution on a 0-50 % linear gradient (100 minutes, 0.8 mL/min) of isopropyl alcohol-acetonitrile (7:3 v/v) containing 0.02 % trifluoroacetic acid by HPLC (column: Capcell-Pak C-18, 4.6 × 150 mm), to fractionate individual fractions.

The partially degraded peptides fractionated and purified were determined of their primary structures with a protein sequence system (Shimadzu, PPSQ-21).

[Preparation of cDNA library]

For the purpose of RNA extraction, the culture broth of Geotrichum candidum Dec 1 strain was subjected to centrifugation, to separate 25 mL of hypha of the strain. This

was placed in a centrifuge tube, followed by addition of liquid nitrogen to freeze the hypha, and then it was freeze-dried for 12 hours to recover a powder.

Over the resulting powder of the hypha was again poured liquid nitrogen, to pulverize the hypha, to which was then added 10 mL of a guanidium solution (containing 4 M guanidium isothiocyanate, 20 mM sodium acetate, pH 5.2, 0.1 mM DTT and 0.5 % N-lauroylsarcosine), for homogenization. This was then centrifuged (1,500 × g) to obtain the supernatant.

The resulting RNA was separated by cesium chloride ultra-centrifugation method (Ullrich), followed by fractionation of poly(A)$^+$ RNA using oligo(dT) cellulose column. The resulting poly(A)$^+$ RNA was used to synthetically prepare cDNA with a cDNA synthesis kit (TaKaRa; Gulbler-Hoffman).

Into the resulting cDNA was inserted an adaptor (EcoRI-NotI-BamHI), by using a DNA ligation kit. Both the ligated termini were phosphorylated with T4 polynucleotide kinase, to separate cDNA by agarose electrophoresis.

Further, 1200- to 2000-bp cDNA corresponding to the molecular weight of DyP was separated among the resulting cDNAs, to which was then added 8 μL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA-2Na) to elute the cDNA.

The recovered cDNA fragment was ligated in the EcoRI site of λ phage. This was packaged into λ phage with Gigapack Gold Packaging Extract (Stratagene, La Jolla Calif., USA).

After E. coli NM514 strain was infected with lambda phage λgt 10 at 37 °C for 15 minutes, the infected strain was overlaid on LB agar medium (bacto-tryptone, 0.5 % bacto-yeast extract, 1 % sodium chloride, 1.5 % agar/1,000 mL), by using 0.7 % agar. The plate was cultured at 37 °C for 12 hours.

[Sequencing of coding gene of dye-degrading enzyme DyP]

Colonies hybridizing with the preliminarily prepared labeled probe were screened. That is to say, by plaque hybridization of the colonies, 11 candidates were consequently selected from the positive cDNA library and DNAs recovered by PCR.

These were ligated with pUC18 plasmid via T4 DNA ligase, for amplification in E. coli JM109 strain.

Because DyP has a molecular weight of 60 kDa at a sugar chain content of 17 %, on the basis of the results of the measurement of the DyP properties, the primary amino acid sequence is estimated to be of 49.8 kDa. Additionally, it is estimated that the open reading frame of the DyP coding gene will comprise 460 amino acids, namely 1380 bp.

Then, the coding genes of the resulting 11 candidates were used for PCR again. Among the inserted cDNAs, a gene around 1380 bp was screened.

Consequently, clone 92 carrying the cDNA of a 1600-bp size was obtained.

By using BamHI, the cDNA was cleaved out of the

recombinant plasmid, which was then inserted in pUC18. The resulting plasmid was designated pB92.

Subsequently, the plasmid DNA was prepared by the alkali extraction method. Both the resulting strands were analyzed and sequenced by a DNA sequencer (Model 4000L, Li-Cor Inc., Lincoln, Neb., USA).

[Expression in a host of different species]

The open reading frame of the pB92 thus recovered comprises 1494 bp, namely 498 amino acids (see SQ ID NO. 7 in the sequence listing). Thus, the molecular weight estimated from the number of the amino acids was 53,306. This indicates that the pB92 carries the DyP gene.

Furthermore, pB92 was transfected into E. coli. The transformant was deposited at the National Institute of Bioscience and Human-Technology, the Ministry of International Trade and Industry, 1-1-3, Higashi, Tsukuba, Ibaraki, Japan. The accession number was FERM BP-7032. The transformant was cultured in L culture medium (0.5 % yeast extract, 0.5 % NaCl, 1.0 % tryptone), and harvested and disrupted, to confirm the DyP activity.

[Comparison with other peroxidase sequences]

Homology screening of the DyP gene was carried out, by using three types of databases (Genebank, EMBL, DDBJ). Consequently, the peroxidase derived from U77073 (Polyporaceae sp.) registered at the Genebank was screened, which was a gene

homologous with DyP. Then, the homology between the two was examined. When regions with high homology were examined, the region at position 407 to position 438 was at the highest homology of 88 %, while the region at position 62 to position 85 was 83 % homologous. For the whole sequence of the gene, only 56 % homology was observed. Additionally, peroxidases with high homology, except for the peroxidase derived from Polyporaceae sp., were never found.

[Comparison with other peroxidases derived from fungi]

Microbial peroxidase is classified in plant-type peroxidase. Plant-type peroxidases are systematically classified into three classes by Welinder et al. (Welinder, Curr. Opin. Struct. Biol., 2, 388-393 (1992)). According to the classification, peroxidases from prokaryote organisms or eukaryote mitochondria are classified in Class I, while fungal peroxidases are classified in Class II and higher plant-derived peroxidases are classified in Class III.

The classification by Welinder et al. is based on the comparison of highly common sequences in the primary sequence of each peroxidase. In more detail, the classification is practiced by comparing the primary sequences around the His residue proximal to the heme iron and the His and Arg residues distal to the heme iron. Using the sequence comparison table prepared by Welinder et al., the DyP sequence was compared (Fig. 4). Herein, Fig. 4 includes CCP (Saccharomyces cerevisiae-

derived cytochrome C peroxidase) and ECP (E. coli-derived peroxidase) as Class I peroxidases. Comparison was done with ARP (Arthromyces ramosus-derived peroxidase), MnP (manganic peroxidase derived from a fungus of the genus Phanerochaete) and LiP (Phanerochaete chrysosporium-derived lignin peroxidase) as Class II peroxidases. Additionally, Class III peroxidase includes TP (Tunip peroxidase) and HRP (horse radish peroxidase).

Because DyP is derived from the fungus, DyP is classified in Class II. However, it was shown that the proximal Arg residue (at the open square (□) position in the figure) characteristically found in Class II was absent and no homology was found with the sequence in the proximity of the distal His residue (at closed circle (●) position in the figure). This indicates that DyP as a fungus-derived peroxidase has a specific sequence.

INDUSTRIAL APPLICABILITY

In accordance with the invention, the peroxidase with high degradation activity of a wide range of dye types as well as the method for degrading the dye types by using the enzyme can be provided. Additionally, the invention provides the genetic information of the enzyme and can supply the enzyme at a large scale on the basis of the information. Thus, the enzyme can be applied to the treatment of wastewater containing dyes

and the like, in the fields of staining industry and the like.

Accordingly, the immobilization of the enzyme as a dye-degrading enzyme can raise the industrial applicability thereof as a bioreactor with higher activity.